

Storage of saposins A and D in infantile neuronal ceroid-lipofuscinosis

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We have isolated storage cytosomes from brain tissue of patients with infantile neuronal ceroid-lipofuscinosis. The purified storage bodies were subjected to compositional analysis which revealed a high content of proteins, accounting for 43% of dry weight. Saposins A and D, also known as sphingolipid activator proteins (SAPs), were shown to constitute a major portion of the accumulated protein using gel electrophoresis and sequence analysis. This is the first time that saposins have been found to be stored in any form of neuronal ceroid-lipofuscinosis.

Infantile neuronal ceroid-lipofuscinosis; Saposin; Lysosomal storage disease; Batten's disease

1. INTRODUCTION

The neuronal ceroid-lipofuscinoses (NCL) [1] collectively constitute the most common group of inherited lysosomal storage disorders of the nervous system, with an estimated incidence of 1:12,500 births [2]. Their clinical manifestations include severe progressive psychomotor deterioration and blindness in the childhood forms [3,4]. Granular autofluorescent sudanophilic storage bodies, which are relatively resistant to lipid solvents in histological sections, accumulate in neurons, and in many other cell types albeit to a lesser extent. The ultrastructure of these electron-dense storage cytosomes varies, being finely granular in the infantile type and displaying a curvilinear or fingerprint pattern in the late infantile and juvenile forms of NCL [5]. Direct protein sequencing has shown that subunit *c* of mitochondrial ATP synthase is stored in an ovine [6] and other animal models of NCL, as well as in the late infantile and juvenile types of human NCL, but not in the infantile form [7]. The genes for the infantile [8] and juvenile [9] types have been mapped to chromosomes 1 and 16, respectively.

Saposins, also called sphingolipid activator proteins (SAPs), are small heat-stable glycoproteins needed for the hydrolysis of sphingolipids in lysosomes [10,11]. They are derived from a common precursor, pro-

saposin, a 70 kDa glycoprotein proteolytically processed to saposins A, B, C and D [12,13]. All saposins are structurally similar to one another, having six conserved cysteines, one common glycosylation site, and prolines in similar positions [11]. In spite of these structural similarities, the individual saposins differ in their specificity for, and mode of activation of, individual sphingolipid hydrolases [14].

Accumulation of saposins has recently been found in certain sphingolipid storage disorders [11,15,16]. We now report the occurrence saposins A and D, as integral constituents of the storage cytosomes in infantile neuronal ceroid-lipofuscinosis (INCL).

2. MATERIALS AND METHODS

2.1. Tissue material

Brain tissue specimens obtained at routine autopsies 5–24 h post-mortem and kept frozen at -70°C from three male and one female INCL patients (ages 11–14 years), one male patient with juvenile neuronal ceroid-lipofuscinosis (JNCL) (age 34 years), and one neurologically normal male individual (37 years of age) were used for the biochemical analyses. The diagnoses of all patients had been verified histologically and by electron microscopy [5].

2.2. Isolation of the storage material

The storage bodies were isolated from the brains of INCL and JNCL patients essentially following the method of Palmer et al. [17]. The tissue was thawed and homogenized in Milli-Q water (1 g/10 ml), then the homogenate was filtered through glass wool and the filtrate sonicated for 1 min and centrifuged at $1,500 \times g$ for 20 min. The resultant pellet was suspended in Milli-Q water, divided into aliquots, loaded onto 20–35% (w/v) CsCl gradients and centrifuged at $20,000 \times g$ for 15 h in a swing-out rotor. The storage material, identified by its characteristic yellow-brownish color, was collected, diluted with Milli-Q water, and pelleted at $1,500 \times g$ for 20 min. All operations were carried out at 4°C . The purity of isolated storage bodies was monitored by electron microscopy. Control brain material was subjected to an identical procedure. Sheep liver storage bodies were purified from sheep affected with ceroid-lipofuscinosis, as described [17].

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Abbreviations: GFAP, glial fibrillary acidic protein; INCL, infantile neuronal ceroid-lipofuscinosis; JNCL, juvenile neuronal ceroid-lipofuscinosis; LDS, lithium dodecyl sulphate; LINCL, late infantile neuronal ceroid-lipofuscinosis; NCL, neuronal ceroid-lipofuscinosis.

2.3. Morphological studies

Tissues fixed in 4% phosphate-buffered formaldehyde were embedded in paraffin and sectioned at 6 μm . Sections were stained for light microscopy using hematoxylin–eosin or Luxol fast blue, Cresyl violet or periodic acid, Schiff or Sudan black B. Unstained sections were viewed with ultraviolet light to detect autofluorescent granules. Small pieces of tissue or isolated storage bodies were fixed in 2% phosphate-buffered glutaraldehyde and embedded in Epon resin for transmission electron microscopy. Double-stained thin sections were viewed in a JEOL-JEM 1200 EX electron microscope at 60 V accelerating voltage.

2.4. Analysis of the composition of the storage cytosomes

Isolated INCL brain storage cytosomes were suspended in Milli-Q water, aliquots dried under vacuum, and then at 60°C until their weights were constant. The weighed storage bodies were then either dissolved in 1% (w/v) lithium dodecyl sulphate (LDS) for protein analyses, or extracted with chloroform:methanol (2:1) for lipid analyses. The amount of protein in storage cytosomes was determined according to Markwell et al. [18], except that sodium dodecyl sulphate (SDS) was replaced with LDS. This method was tested for its compatibility with LDS using bovine serum albumin and β -lactoglobulin as standards. β -Lactoglobulin was used as the quantitative standard in subsequent analyses. Values determined for samples from individual patients were within 13% of the mean.

Lipids from storage bodies were extracted with chloroform:methanol (2:1) overnight at room temperature, 0.2 vol. of Milli-Q water added, and the phases separated by centrifugation at $2,000 \times g$ for 10 min. The lipid-containing lower phase was weighed after careful drying at 60°C.

2.5. Polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

The protein components in the stored material were analyzed on PAGE. Both 15% Laemmli [19] and 16.5% glycerol-containing Schagger [20] gels were used. LDS, rather than SDS, was used as a detergent in sample buffers, and in Schagger gels. The proteins on gels were stained either with Coomassie brilliant blue R or with a double silver staining method that visualizes proteolipids [21].

Western blotting was performed according to Towbin et al. [22], except that the Immun-Blot Alkaline Phosphatase Assay Kit (Bio-Rad Laboratories, Richmond, CA) was used to visualize the immunoreactive proteins. GFAP antiserum was purchased from DAKO (Copenhagen, Denmark). Antibodies were raised in rabbits against subunit *c* purified from liver storage bodies of sheep affected with NCL. The antigen was dissolved into the RIBI adjuvant system (RIBI Immunochem Research Inc., Hamilton, MT), and 500 μg injected 4 times at monthly intervals according to the manufacturer's instructions.

2.6. Protein sequence analysis

All amino acid sequence analyses were performed with a fully automated ABI 477A/120A protein/peptide sequencer using Edman degradation followed by on-line analysis of released phenyl thiohydantoin amino acid derivatives. Proteins were electroblotted onto polyvinylidene difluoride (Immobilon P; Millipore, Bedford, MA) membranes after PAGE. The blots were briefly stained with 0.2% (w/v) Coomassie brilliant blue R in 40% (v/v) methanol, 0.5% (v/v) acetic acid, and destained with 50% (v/v) methanol in water. After staining, the desired bands were cut out and sequenced as described [23]. Sequence analysis was also performed directly from purified storage bodies suspended in Milli-Q water by applying them to a Polybrene-containing (Fluka Chemie AG, Buchs, Switzerland) trifluoroacetic acid-treated glass-fibre filter.

3. RESULTS

3.1. Purity and ultrastructure of storage bodies

The purified storage material from INCL brain tissue was composed almost entirely of conglomerates of

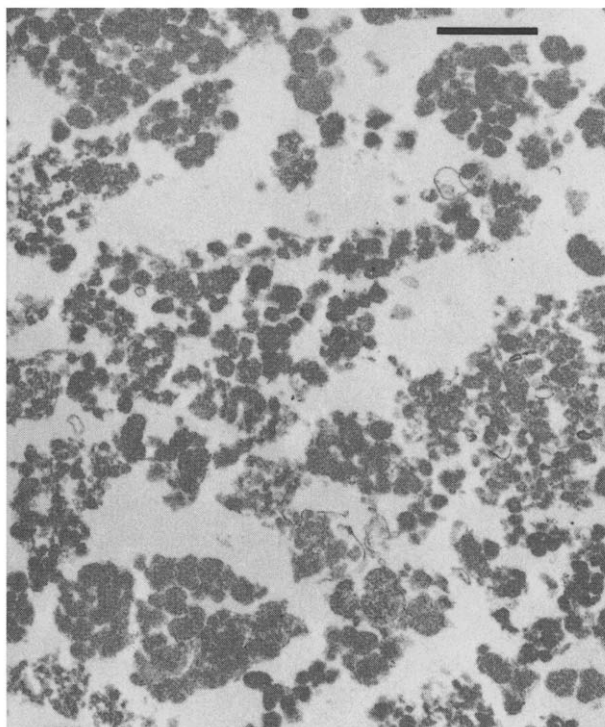


Fig. 1 Purified storage material, isolated from brain tissue of an INCL patient, showing conglomerates of spherical electron-dense cytosomes with a granular internal structure. Electron micrograph: bar = 1 μm .

spherical electron dense cytosomes with a finely granular ultrastructure (Fig. 1), corresponding to characteristic cytoplasmic storage bodies, as seen in intact tissues of INCL patients [24,25]. Occasional mitochondria constituted the only detectable impurity. An identical isolation procedure applied to control brain tissue did not yield any material.

3.2. Composition of the storage cytosomes

Two to four preparations were used to measure the amount of protein in the storage material from each of four patients. Proteins accounted for 43% on average of storage body dry weight. Lipids were measured from two patients and accounted for 35% on average of storage body dry weight. Silver staining of the lipid extract after PAGE showed that there were no proteins in this fraction.

3.3. Proteins in the storage material

Direct sequencing of the INCL storage material of three patients revealed the presence of two major proteins in the storage cytosomes, in approximately equimolar amounts. The sequences of residues 1–25 were determined. These sequences were also present in bands excised from blots, sometimes in different amounts (see below) which allowed the construction of probable fragments of individual sequences. The data were then untangled into two clear sequences by com-



Fig. 2. N-Terminal amino acid sequences obtained by direct sequencing of isolated storage bodies, compared with the sequences of saposins A and D. An asterisk indicates a glycosylation site, an X indicates an unidentified amino acid, and a dash indicates a cysteine or a glycosylated asparagine which did not produce a signal.

parison with the known sequences of the saposins [11]. One was found to be identical with the N-terminal sequence of saposin A, and the other to saposin D, which is truncated by two amino acids from the sequence deduced from the cDNA (Fig. 2). Cysteine residues, as well as the glycosylated asparagine residue, did not produce signals in the sequence analysis.

The protein components of the purified brain storage cytosomes were further analyzed on both Laemmli and Schagger gels using Coomassie blue and double silver staining methods. The results were essentially the same in all four patients and in all systems, showing one major and several minor protein bands (Fig. 3). The major band had an apparent molecular weight of 14 kDa. N-Terminal sequence analysis showed the presence of both saposin A and saposin D in the same band.

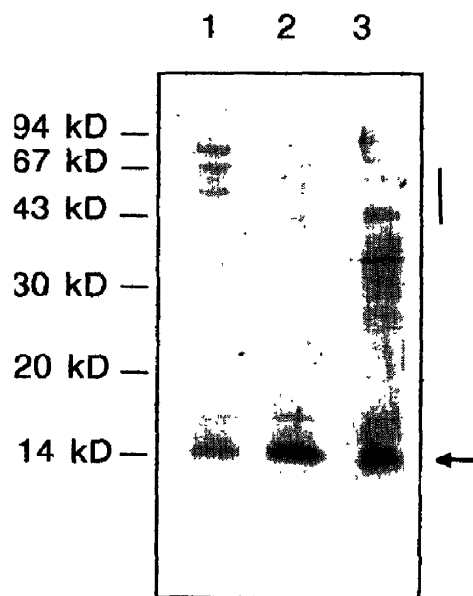


Fig. 3. Storage bodies isolated from two INCL patients analyzed on SDS-PAGE: patient 1 (lane 1), patient 2 (lanes 2 and 3). Lanes 1 and 2 were stained with Coomassie brilliant blue R, and lane 3 was stained with the double silver stain. The arrow indicates the major band containing saposins A and D, as shown by N-terminal sequence analysis. The bar indicates proteins showing immunoreactivity against GFAP antiserum in Western blotting.

Although saposins A and D differ by 3 kDa in molecular weight [14], they are known to overlap in gels, being separable only by high-pressure liquid chromatography using a two column system [11].

Minor bands from 20 to 35 kDa in molecular weight were also subjected to N-terminal sequence analysis. The bands contained both saposin A and saposin D in varying proportions in the different bands, and may represent aggregated saposins.

A group of higher molecular weight proteins (35–50 kDa) were shown to be of glial origin. N-Terminal sequence analysis of a 37 kDa protein showed identity with glial fibrillary acidic protein (GFAP). However, the N-terminus of this 37 kDa protein was found inside GFAP, which, together with the fact that the molecular weight of GFAP is approximately 50 kDa [26], suggests that GFAP had been partially degraded in the samples. Furthermore, this 37 kDa band and several others of higher molecular weight (indicated by a bar in Fig. 3) reacted with a commercial GFAP-antibody in Western blotting. However, the GFAP fragment does not seem to be an integral storage body component. Highly elevated amounts of GFAP in INCL brain has been reported before [27], and may have caused the contamination of storage bodies with a small amount of this protein.

The absence of mitochondrial ATP synthase subunit *c* in the INCL storage material was confirmed by Western analysis using subunit *c* antiserum (Fig. 4). Storage bodies purified from liver of sheep affected with NCL, and storage bodies purified from brain tissue of a JNCL patient, were used as positive controls, giving two signals corresponding to monomeric and oligomeric forms of subunit *c* (3.5 and 14.8 kDa in apparent molecular weight). Storage bodies purified from brain tissue of INCL patients did not show reactivity against subunit *c* antiserum.

4. DISCUSSION

The basic biochemical defect remains unknown in all forms of NCL, despite a wide variety of hypotheses that have been advanced. These include abnormal peroxida-

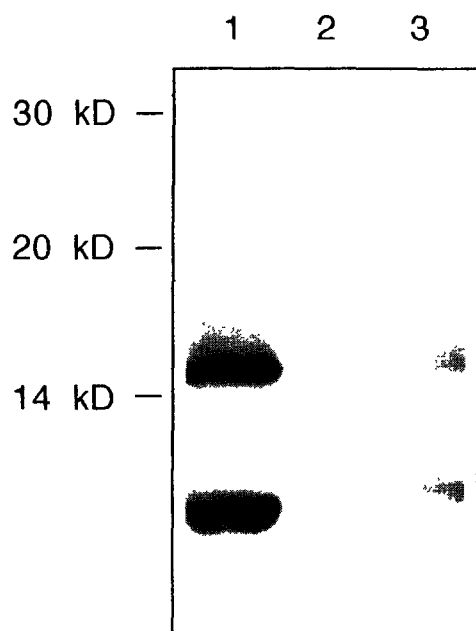


Fig. 4. Western blot using antiserum raised against subunit *c* of mitochondrial ATP synthase. Storage bodies isolated from liver of sheep affected with NCL (lane 1), brain of INCL patient (lane 2), and brain of JNCL patient (lane 3). The two immunoreactive bands in lanes 1 and 3 correspond to monomeric and oligomeric forms of subunit *c*, respectively. Note the lack of immunoreactivity of the INCL storage bodies.

tion of lipids [28,29], deficiency of a peroxidase [30], and disturbances in the metabolism of fatty acids [31,32], iron [33], retinoic acid, dolichols [34,35], and dolichol-linked oligosaccharides [36,37]. Further suggestions include a defective thiol endoprotease causing faulty recycling of lysosomal membranes [38], inhibition of cathepsin B activity by accumulating abnormal peroxides [39], a defect in very low density lipoprotein synthesis [40], and abnormal methionine S-methylation [41]. None of these have been substantiated.

Recent studies of ovine NCL have shown that the storage cytosomes largely consist of protein with one major component, subunit *c* of mitochondrial ATP synthase [6] with a normal amino acid sequence [21]. Accumulation of subunit *c* of mitochondrial ATP synthase has also been found in the bovine and certain canine forms of NCL, as well as in the human late infantile neuronal ceroid-lipofuscinosis (LINCL) and JNCL [7]. The primary defects causing these accumulations of subunit *c* are still unknown, but may involve a specific degradation pathway for subunit *c* [7].

Our results confirm the earlier observations [7] that subunit *c* is not enriched in the INCL storage bodies. The present study also revealed a high protein content (43% of dry weight) in storage bodies derived from INCL brain, and indicate that saposins A and D constitute a major portion of the accumulated protein. The electrophoretic behaviour of these molecules suggest

that the complete molecules, or at least substantial portions of them, are stored. N-Terminal truncation of the cDNA-deduced sequence of saposin D is normal and is not specific for INCL [42].

Recent discoveries of mutations in the prosaposin gene have demonstrated the essential role of saposins in sphingolipid hydrolysis in human tissues [43–45]. However, primary disturbances of saposin metabolism have not been reported to be associated with storage of saposins [43]. It is also of interest in this context to note that no accumulation of saposins was observed in LINCL [15] when entire brain tissue was analyzed. In contrast, an up to 80-fold accumulation of saposins A, B, C, and D has been found in spleen and/or liver of patients with Gaucher disease, type I Niemann-Pick disease, and fucosidosis, as well as in brain of patients with Tay–Sachs and infantile Sandhoff diseases [11,15,16]. These are all primary disturbances of sphingolipid metabolism with established enzyme defects, and consequently the accumulation of saposins seems to be a secondary feature.

Two alternative hypotheses have been advanced to explain the accumulation of saposins in the sphingolipid storage diseases [14]. Firstly, synthesis of saposins may be stimulated by the accumulation of a defective target hydrolase or lipid as a compensatory mechanism. Secondly, saposins may be co-deposited with the accumulating sphingolipids. Available experimental data strongly favor the latter hypothesis. Parallel accumulation of ganglioside GM₂ and saposin A has been observed in Tay–Sachs and Sandhoff diseases, glucocerebroside and saposins A and D in Gaucher disease, and fucoglycolipid and saposins A and D in fucosidosis [15]. Furthermore, loading of sulfatides into cultured fibroblasts from a metachromatic leucodystrophy patient has been shown to result in simultaneous lysosomal accumulation of sulfatides and saposin B [46], and the amount of saposin C has been reported to increase in mouse tissues after injection of glucocerebroside [47].

Our present study shows the accumulation of saposins A and D, together with lipids, in the storage cytosomes from brains of INCL patients. The different chromosomal localizations of the INCL gene (chromosome 1) and prosaposin gene (chromosome 10), indicate that the accumulation of saposins in INCL is likely to be a secondary phenomenon. However, the protein-to-lipid ratio (>1) in the storage material of INCL patients is much higher than that observed in, for example, Tay–Sachs disease, where the isolated cytoplasmic membranous bodies contain only about 10% protein on a weight basis [48], and the actual quantity of saposin A is lower than GM₂ ganglioside by an order of magnitude [15]. Additional studies will have to be carried out to fully characterize the stored saposins and the mechanism of saposin accumulation in INCL, issues likely to be important for the understanding of the pathogenesis of this disease.

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